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Differential gene expression in egg cells and zygotes suggests that the transcriptome is restructed before the first zygotic division in tobacco

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Abstract **We applied suppression subtractive hybridization and mirror orientation selection to compare gene expression profiles of isolated *Nicotiana tabacum* cv SR1 zygotes and egg cells. Our results revealed that many differentially expressed genes in zygotes were transcribed de novo after fertilization. Some of these genes are critical to zygote polarity and pattern formation during early embryogenesis. This suggests that the transcriptome is restructed in zygote and that the maternal-to-zygotic transition happens before the first zygotic division, which is much earlier in higher plants than in animals. The expressed sequence tags used in this study provide a valuable resource for future research on fertilization and early embryogenesis.**

Keywords: Egg cell; Fertilization; Gene expression; Mirror orientation selection; Suppression subtractive hybridization; Zygote; Zygotic gene activation

1. Introduction

Fertilization occurs when a sperm penetrates an egg cell. The fertilized egg cell, or zygote (one-cell embryo), then undergoes a series of complex morphological and physiological changes. Among these changes, a critical event for the onset of early embryogenesis is zygotic gene activation (ZGA), which denotes the de novo transcription at the beginning of embryogenesis. In animals, the timing of ZGA is regulated by a "zygotic clock" and is somewhat species-dependent. The established theory is that the unfertilized egg cells of animals store abundant mRNAs encoding the proteins that sustain early embryo development, and the onset of global ZGA occurs much later after fertilization, even up to the 16-cell embryo stage [1–4].

In contrast to animals, fertilization and embryogenesis in higher plants occur deep within the maternal tissues of the ovule. Although several laboratories have worked with a variety of plant species, little is known about the timing of ZGA and maternal-to-zygotic transition during fertilization and early embryogenesis because of the difficulty in accessing female gametes, zygotes, and early embryos. Microarray analyses of precocious embryonic development in apomictic hybrids between maize and its wild relative *Tripsacum* have shown that early embryo development occurs without significant changes in the transcript population in the unfertilized ovule of sexual maize, suggesting that the maternal-to-zygotic transition does not occur until at least 3 days after fertilization when proembryos have already formed [5].

Different evidence has also been presented. Fertilization of wild-type egg cells with transgenic pollen results in detectable GUS activity during early embryogenesis in *Arabidopsis* [6,7]. In maize, a transgene driven by a 35S promoter in the paternal genome is almost immediately transcribed and translated in the zygote [8]. In *Arabidopsis*, an investigation of the timing of transgene activation after fertilization has also shown de novo GUS activity in zygotes [9]. An analysis of individual gene expression in in vitro fertilized egg cells of maize indicated that ZGA occurs earlier in plants than in animal systems [10,11]. Genes upregulated in the apical or basal cells after in vitro fertilization are already expressed in the early zygotes, but not in egg cells, implying that expression is initiated in maize zygotes [12]. As the only example, a global study of the transcript profile of wheat egg cells and two-celled proembryos has shown that the transcription composition of two-celled proembryos is distinct from that of egg cells [13]. To date, most available data have been from various studies of individual genes. These distinct data do not present an overview of differentially expressed genes in fertilized egg cells. Also, to our knowledge, there is no direct evidence showing that endogenous genes are actively and widely transcribed in zygotes of dicotyledonous plants.

To better understand the molecular regulation of fertilization, we have developed reliable techniques to isolate healthy eggs and zygotes from tobacco to investigate gene expression in egg cells before and after in vivo fertilization. In this study, we constructed tobacco egg and zygote cDNA libraries and contrasted their mRNA expression profiles to reveal genes that are differentially expressed using suppression subtractive hybridization (SSH) [14,15]. To identify truly differentially expressed genes, we used mirror orientation selection (MOS), which is efficient for eliminating background molecules in the subtracted library [16].

2. Materials and methods

2.1. Isolation of eggs, zygotes and sperm cells

Egg cells were isolated from tobacco (*Nicotiana tabacum* cv SR1) ovules 40 h after pollination (HAP) as previously described [17], and zygotes were isolated from ovules 96 HAP using a method of enzymatic maceration combined with grinding [18]. Sperm cells were isolated as described [19]. To inhibit the modulation of gene expression in response to possible stresses during physical isolation and enzymatic

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digestion, two transcription inhibitors, actinomycin D (50 mg/L) and cordycepin (100 mg/L), were applied to all solutions in the isolation process. Single cells were transferred to 2× lysis/binding buffer in 0.2-ml tubes and frozen immediately in liquid nitrogen for later use.

2.2. mRNA isolation and cDNA synthesis

Sixty-one egg cells and zygotes, respectively, to be used for SSH were lysed, and mRNA was isolated using a Dynalbeads mRNA DIRECT Micro kit (Dynal Biotech, Oslo, Norway), following the manufacturer's instructions (although the annealing volume was reduced to 50 μl for semiquantitative RT-PCR). A SMART cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA) was used to reverse-transcribe mRNA isolated from eggs and zygotes and amplify cDNA. Each individual amplification required 23 PCR cycles.

2.3. Suppression subtractive hybridization and mirror orientation selection

SSH was performed with a PCR-Select cDNA Subtraction kit (Clontech) according to the manufacturer's recommendations but with modifications for MOS [16]. MOS cDNA was cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into DH5α for differential screening.

2.4. Reverse cDNA dot-blot analysis

Clones obtained by SSH and MOS were further analyzed by dot-blot analysis. Randomly selected clones were arrayed in 96-well microtiter dishes with 200 μl of Luria–Bertani broth containing ampicillin and cultured overnight on a shaker. We used 1 μl of the culture for PCR with the primer NP2Rs (5′-GGTCGCGGCCGAGGT-3′). PCR products were spotted on positively charged nylon membranes (Roche Molecular Biochemicals, Mannheim, Germany). DIG-labeled DNA probes were generated by a DIG DNA Labeling Kit (Roche), following the manufacturer's instructions but using a random nonamer mix instead of hexanucleotides. Hybridization and screening were conducted as described previously [15]. Putative differential clones were selected for DNA sequencing.

2.5. DNA sequence analysis

Insert-DNA sequencing was performed on ABI3730 machines (PE–Applied Biosystems, Foster City, CA, USA). Vector and adaptor sequences were trimmed using Vector NTI Suite 8.0 (Informax, North Bethesda, MD, USA) prior to further analysis. Chimeric cDNA sequences containing transcripts from different genes [20] were eliminated. The sequences were clustered using CAP3 [21]. Groups that contained only one sequence were classified as singletons. To assign functions, the assembled consensus sequences and valid expressed sequence tags (ESTs) were used as a query in BLASTN (non-redundant database and EST_others database) and BLASTX (non-redundant database and SwissProt databases) searches [22–24] of the National Center for Biotechnology Information (NCBI) Web Service Sequence (http://www.ncbi.nlm.nih.gov).

2.6. Semiquantitative RT-PCR

Putative differentially expressed cDNA clones that yielded strong positive signals in differential screening analysis were selected for semi-quantitative RT-PCR. The mRNA of sperm cells (400 cells), egg cells and zygotes (20 cells each) were isolated using a Dynalbeads mRNA DIRECT Micro kit as described above, and first-strand cDNA synthesis was performed directly with mRNA bound to the magnetic beads, as recommended by the manufacturer. First-strand cDNA was synthesized in a 10-μl reaction volume containing 100 U SuperScriptIIReverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The quality and quantity of cDNAs were tested using PCR with primers for the tobacco *G3PDH* gene (Accession No. AJ133422 G3PDHS: 5′-GGCTGTTACTGTTTTTGGCTTTA-3′, G3PDHAS1: 5′-TCGGGCTTGTATTCTTTCTCATT-3′) and *actin* gene (Accession No. X63603, ACTINS: 5′-CAAGGCAGGGTTTGCTGGAGATG-3′, ACTINA: 5′-GTCGAACCGCCACTGAGTACAAT-3′). Specific oligonucleotides were designed for each chosen cDNA using Primer Premier (Premier Biosoft International, Palo Alto, CA, USA) and used in sets for PCR. The PCR conditions were 30–39 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. Each reaction was repeated with another batch of cDNA. After separation on 2% (w/v) agarose gels, the intensities of the same DNA products were compared between reactions from different templates.

3. Results and discussion

3.1. Isolation of egg cells and zygotes from the female gametophyte of tobacco

In tobacco, the time course of fertilization and the first division are variable. Fertilization takes place around 45–48 HAP, and the first division of in vivo zygotes occurs around 120 HAP [18]. The long time interval between fertilization and the first cell division makes tobacco an ideal model plant to investigate spatial and temporal patterns of ZGA. For this study, we chose unfertilized mature egg cells from ovules 40 HAP and zygotes from ovules 96 HAP. A well established method based on enzymatic maceration combined with brief grinding [17,18] allowed us to isolate enough egg cells and zygotes for cDNA synthesis. To inhibit the modulation of gene expression in response to possible stresses during physical isolation and enzymatic digestions for egg cells and zygotes, two transcription inhibitors, actinomycin D and cordycepin, which have been proven effective in suppressing the induction of stress-inducible genes [25], were applied during all steps of cell isolation. After the treatment and isolation procedure, the egg cells and zygotes were viable and healthy (Fig. 1).

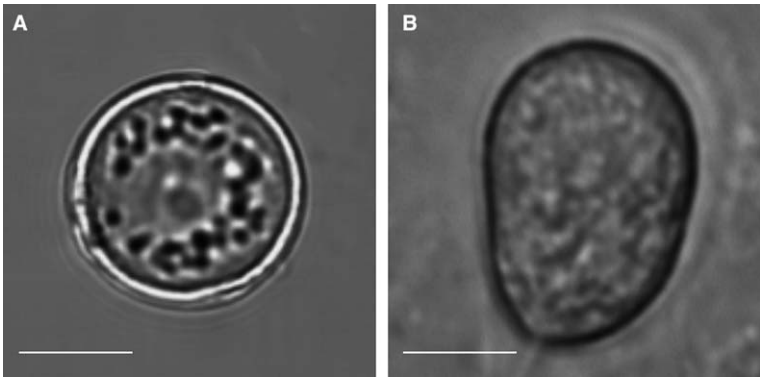

Fig. 1. Isolated egg cell and zygote from a female gametophyte of tobacco. (A) Isolated egg cell from an ovule 40 HAP. (B) Isolated zygote from an ovule 96 HAP. Scale bar = 10 μM.

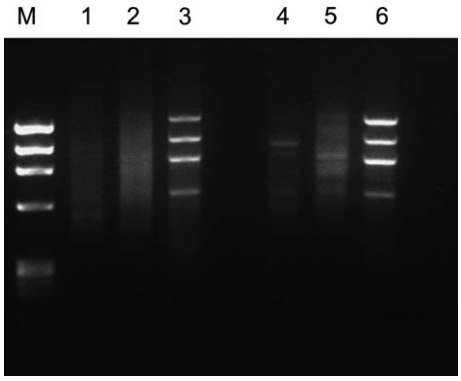

Fig. 2. Gel electrophoresis of PCR products after application of SSH and MOS technique to the subtraction of egg cell and zygote cDNA. LaneM: Φx174/HaeIII. Lane 1: results of applying SSH procedures to egg/zygote (egg cell cDNA as tester) subtraction. Lane 2: results of applying SSH procedures to zygote/egg subtraction after SSH. Lane 4: MOS-generated sample of egg/zygote. Lane 5: MOS-generated sample of zygote/egg. Lanes 3 and 6: samples of control subtraction, egg cell cDNA containing ΦX174/HaeIII DNA as tester cDNA and egg cell cDNA as driver cDNA, each fragment in Φx174/HaeIII corresponds about 0.01% of the total cDNA.

3.2. Identification of differentially expressed genes

We compared cDNA profiles of egg cell and zygote via SSH and MOS. SSH is a highly effective method for generation of subtracted cDNA libraries [14,15]. One of drawbacks of SSH, however, is the presence of background molecules in the subtracted library. These cDNAs give positive results in the differential screening process, but they cannot be confirmed by independent experiments like Northern blot hybridizations [16]. Mirror orientation selection(MOS), a simple procedure that substantially decreases the number of background clones in the SSH-generated libraries [16], was performed to eliminate false positive clones. As shown in Fig. 2, the application of MOS led to a reduction of smear cDNAs, and appearance of recognizable bands which indicated elimination of background molecules and enrichment of differentially expressed genes.

In this study, we developed two subtracted cDNA libraries that are useful in the identification of differentially expressed genes before and after fertilization. In total, we screened 184 and 460 clones from egg and zygote subtracted libraries, respectively, by cDNA dot-blot hybridization according to the established method [15]. A set of 63 clones from the forward library and 86 from the reverse library was selected based on differential intensities of the hybridization signals and then sequenced. The cDNA sequences of each library were clustered and assembled into contigs. Nineteen clusters from the egg cells were assembled from 63 ESTs in the form of 10 clusters containing 2 or more ESTs and 9 singletons, while 86 ESTs from zygotes formed 42 clusters including 24 singletons. The consensus sequence from clusters containing 2 or more ESTs or the valid sequence of singletons was compared to sequences in the database using the BLASTX and BLASTN program [22–24]. ESTs with a significant match (*E*-value $<10^{-5}$) were assigned putative identities based on a BLASTX search against the non-redundant protein database in GenBank. Differential expression of clusters with similarity to known sequences in public databases was validated by semiquantitative RT-PCR. For clusters that had more than one EST, the consensus sequence was chosen. Two constitutively expressed genes, G3PDH and *Actin*, which exhibited nearly identical expression levels in egg cells and zygotes, were used as controls. Table 1 lists selected EST clusters confirmed by semiquantitative RT-PCR.

3.3. Differentially or specifically expressed genes

The most abundant ESTs found in the subtracted egg cDNA library were members of ECA1-like genes of unknown function, which are expressed specifically in embryogenic microspore cultures [26]. Thirty-six (57%) of the 63 ESTs in the subtracted egg cell cDNA library were ECA1-like genes. ECA1-like transcripts are also the largest EST group in wheat egg cells [13,27]. They encode small proteins with a putative signal peptide for extracellular localization. The signal appears slightly downregulated in zygotes (Fig. 3).

Transcripts coding for annexin were also found to be reduced in zygotes. Annexins are Ca^{2+}- and phospholipid-binding proteins that form an evolutionarily conserved multigene family with members of the family being expressed throughout the animal and plant kingdoms. Extensive studies of these proteins in animal cells have shown their multifunctional roles in essential cellular processes such as membrane trafficking, ion transport, mitotic signaling, cytoskeleton rearrangement, and DNA replication [28]. The functions of annexins have been determined in several plant species [29–34]. The fact that plant annexins are highly expressed in secretory cells, such as the outer cells of root caps, epidermal cells, and developing xylem and phloem cells, suggests that annexins function in Ca^{2+}-stimulated exocytosis and Golgi-mediated secretion of plasma membrane and wall materials in plant cells [29–34]. Annexin p35 is also abundantly stored in egg cells of maize [35]. Annexins in egg cells and zygotes may play a role in exocytosis after fertilization as well as in plasma-membrane and cell-wall formation in the zygote.

Transcripts of ZC35 encode a protein that is homologous to the transcription factor *WUSCHEL-related homeobox 9* (*WOX9*) in *Arabidopsis*. *WOX9* is first detected in basal cells and subsequently becomes restricted to the hypophysis; at the 8-cell stage, its expression expands to the central domain of the embryo in response to signaling from the embryo proper [36]. In *Arabidopsis*, *WOX9* was not detected in egg cells and zygotes. We also did not observe ZC35 in egg cells, but found that it was expressed in zygotes (Fig. 3). This suggests that *WOX9* is already present in zygotes before the first division and may be involved in zygote polarity related to future cell fate determination after the first zygotic division.

A transcript for a protein similar to embryo-abundant protein (PgEMB34) in *Picea glauca* was also transcribed de novo in zygotes. PgEMB34 is abundant in immature somatic embryos and gradually decreases after transition to globular and cotyledonary stages. Transcripts homologous to PgEMB34 have also been detected at equivalent developmental stages in zygotic embryos [37]. In our experiments, it was detected in zygotes (Fig. 3), but not in egg cells, suggesting that it is actively transcribed after fertilization and may play a role in early embryo development.

3.4. Zygotic gene activation occurs at the one-cell stage in tobacco

In this study, semiquantitative RT-PCR confirmed that 9 clusters (Class vi in Table 1) are present in zygotes and never

Table 1
Differentially expressed genes with similarity to known sequences

Cluster ID	Number of clones	Protein homologue (Accession No.)[a]	*E*-value
(i) Clusters at a reduced level in the zygote relative to the egg, present in the sperm			
EC1[b]	13	*Arabidopsis thaliana* hypothetical protein (CAB80597)	7e – 24
(ii) Clusters at a reduced level in the zygote relative to the egg, absent in the sperm			
EC2	5	*Glycine max* 60S acidic ribosomal protein P0 (P50346)	2e – 124
EC11	1	*Nicotiana tabacum* p32.2 annexin (CAA76770)	2e – 89
EC15	1	*Lycopersicon esculentum* 1-aminocyclopropane-1-carboxylate oxidase (BAA34924)	5e – 146
(iii) Clusters present in the egg and at an enhanced level in the zygote, present in the sperm			
ZC10	2	*Solanum tuberosum* pyrophosphate–fructose 6-phosphate 1-phosphotransferase α subunit (P21342)	4e – 154
(iv) Clusters present in the egg and at an enhanced level in the zygote, absent in the sperm			
ZC5	2	*Nicotiana tabacum* lipoxygenase (CAA58859)	4e – 73
ZC17	3	*Nicotiana tabacum* H2A histone (BAC53941)	3e – 39
(v) Clusters absent in the egg, present in the sperm and zygote			
ZC18	13	*Nicotiana tabacum* α-tubulin (CAD13177)	2e – 126
ZC26	1	*Theobroma cacao* carboxypeptidase type III (CAC86383)	3e – 86
ZC36	1	*Arabidopsis thaliana* glutamic acid-rich protein (BAD93796)	3e – 14
(vi) Clusters absent in the sperm and egg, present in the zygote			
ZC1	9	*Arabidopsis thaliana* dynamin family protein (NP_176252)	5e – 52
ZC7	3	*Nicotiana tabacum* chromomethylase-like protein (BAC53936)	5e – 122
ZC8	2	*Lycopersicon esculentum* putative glucosyltransferase (AAL92461)	7e – 78
ZC11	2	*Nicotiana tabacum* endo-β-1,4-glucanase precursor (AAL30452)	2e – 131
ZC19	1	*Arabidopsis thaliana* Msh6-2 protein (CAA07685)	2e – 81
ZC21	1	*Pisum sativum* embryo-abundant protein EMB (AAM19356)	1e – 58
ZC28	1	*Lycopersicon esculentum* DNA repair protein RAD51 homolog (Q40134)	7e – 90
ZC32	1	*Arabidopsis thaliana* chloroplast nucleoid DNA binding protein (AAM66061)	4e – 72
ZC35	1	*Arabidopsis thaliana* WOX9 protein (Q6X7J4)	5e – 34

[a]Maximum homology is obtained using BLASTX against the NCBI's nr database.
[b]Shows significant similarity with *ECA1* from barley. At least five different clusters have significant similarity with *ECA1* in subtracted egg cell cDNA library.

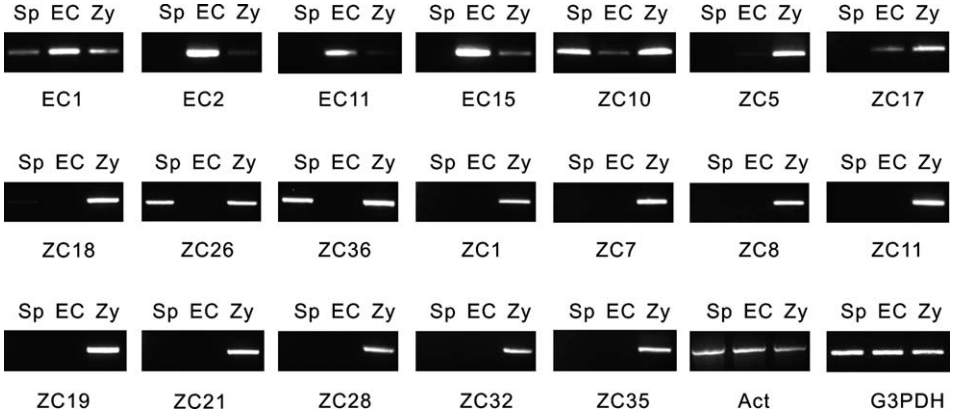

Fig. 3. Semiquantitative RT-PCR of transcripts differentially expressed in egg cells and zygotes. Sperms (Sp), egg cells (EC)and zygotes (Zy) cDNA were used as templates. Samples were normalized using the housekeeping genes G3PDH and actin (Act), which did not show differential signals between egg cells and zygotes.

detected in egg cells and sperm cells (Fig. 3), indicating that they were synthesized de novo in tobacco zygotes and suggesting that the zygotic genome was actively transcribed after fertilization and before the first cell division. The fact that several transcripts with similarity to embryo-specific genes were found in zygotes also provides an additional line of evidence for maternal-to-zygotic transition taking place in the zygote. This is consistent with the onset of ZGA previously reported during early embryogenesis [8–13]. Our results also imply that a reprogramming of gene expression occurs in zygotes, which leads to a developmental program under embryonic control. However, our results is different from the data reported in a study on maize, which indicated no significant difference between mRNA populations from ovules containing an egg cell or a proembryo and suggested that the first divisions in the proembryo likely occur before the onset of global embryo genome activation [5]. This difference may be attributable to the different mechanisms of fertilization and early embryogenesis

between dicotyledons and monocotyledons, or that the ZGA in higher plants is species-dependent, as in animals.

As previously revealed, sperm cells may have a diverse complement of mRNAs [38,39]. In our results (Fig. 3), there are 4 clusters that are present in both sperm cells and zygotes. It is not clear yet whether they are coming from sperm cytoplasm or transcribed de novo after fertilization. It remains to be shown whether the transcripts synthesized de novo in zygotes are maternal, paternal, or both. The availability of EST data from this study generates molecular and genetic markers and creates the possibility of conducting additional investigations on expression patterns of de novo transcribed genes in zygotes with regard to the parental origin.

In conclusion, we favor the assumption that ZGA occurs earlier in higher plants than in animals. Some important genes specifically expressed in embryos and closely related to specific development events of embryogenesis suggest that the maternal-to-zygotic transition may occur before the first zygotic division in higher plants. More generally, characterizing these genes and studying the potential roles of differentially expressed genes will promote promising research on molecular mechanisms of fertilization and early embryogenesis.

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References

[1] Nothias, J.Y., Majumder, S., Kaneko, K.J. and DePamphilis, M.L. (1995) Regulation of gene expression at the beginning of mammalian development. J. Biol. Chem. 270, 22077–22080.
[2] Schultz, R.M. (1993) Regulation of zygotic gene activation in the mouse. Bioessays 15, 531–538.
[3] Ram, P.T. and Schultz, R.M. (1993) Reporter gene expression in G2 of the 1-cell mouse embryo. Dev. Biol. 156, 552–556.
[4] Christians, E., Rao, V.H. and Renard, J.P. (1994) Sequential acquisition of transcriptional control during early embryonic development in the rabbit. Dev. Biol. 164, 160–172.
[5] Grimanelli, D., Perotti, E., Ramirez, J. and Leblanc, O. (2005) Timing of the maternal-to-zygotic transition during early seed development in maize. Plant Cell 17, 1061–1072.
[6] Weijers, D., Geldner, N., Offringa, R. and Jurgens, G. (2001) Seed development: early paternal gene activity in Arabidopsis. Nature 414, 709–710.
[7] Kohler, C., Page, D.R., Gagliardini, V. and Grossniklaus, U. (2005) The *Arabidopsis thaliana* MEDEA polycomb group protein controls expression of PHERES1 by parental imprinting. Nat. Genet. 37, 28–30.
[8] Scholten, S., Lorz, H. and Kranz, E. (2002) Paternal mRNA and protein synthesis coincides with male chromatin decondensation in maize zygotes. Plant J. 32, 221–231.
[9] Baroux, C., Blanvillain, R. and Gallois, P. (2001) Paternally inherited transgenes are down-regulated but retain low activity during early embryogenesis in Arabidopsis. FEBS Lett. 509, 11–16.
[10] Dresselhaus, T., Cordts, S., Heuer, S., Sauter, M., Lorz, H. and Kranz, E. (1999) Novel ribosomal genes from maize are differentially expressed in the zygotic and somatic cell cycles. Mol. Gen. Genet. 261, 416–427.
[11] Sauter, M., von Wiegen, P., Lorz, H. and Kranz, E. (1998) Cell cycle regulatory genes from maize are differentially controlled during fertilization and first embryonic cell division. Sex. Plant Reprod. 11, 41–48.
[12] Okamoto, T., Scholten, S., Lorz, H. and Kranz, E. (2005) Identification of genes that are up- or down-regulated in the apical or basal cell of maize two-celled embryos and monitoring their expression during zygote development by a cell manipulation- and PCR-based approach. Plant Cell Physiol. 46, 332–338.
[13] Sprunck, S., Baumann, U., Edwards, K., Langridge, P. and Dresselhaus, T. (2005) The transcript composition of egg cells changes significantly following fertilization in wheat (*Triticum aestivum* L.). Plant J. 41, 660–672.
[14] Diatchenko, L. et al. (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. USA 93, 6025–6030.
[15] Diatchenko, L., Lukyanov, S., Lau, Y.F. and Siebert, P.D. (1999) Suppression subtractive hybridization: a versatile method for identifying differentially expressed genes. Methods Enzymol. 303, 349–380.
[16] Rebrikov, D.V., Britanova, O.V., Gurskaya, N.G., Lukyanov, K.A., Tarabykin, V.S. and Lukyanov, S.A. (2000) Mirror orientation selection (MOS): a method for eliminating false positive clones from libraries generated by suppression subtractive hybridization. Nucleic Acids Res. 28, E90.
[17] Sun, M.-X., Yang, H.-Y. and Zhou, C. (1993) A new method for embryo sac isolation and in situ fusion of egg and synergid protoplasts in *Nicotiana tabacum*. Acta Bot. Sin. 35, 893–900.
[18] Fu, C.-M., Sun, M.-X., Zhou, C. and Yang, H.-Y. (1996) Isolation of fertilized embryo sacs and zygotes and triggering of zygote division in vitro in *Nicotiana tabacum*. Acta Bot. Sin. 38, 262–267.
[19] Sun, M.-X., Moscatelli, A., Yang, H.-Y. and Cresti, M. (2000) In vitro double fertilization in *Nicotiana tabacum* (L.): fusion behavior and gamete interaction traced by video-enhanced microscopy. Sex. Plant Reprod. 12, 267–275.
[20] Zhang, J., Underwood, L.E. and D'Ercole, A.J. (2000) Formation of chimeric cDNAs during suppression subtractive hybridization and subsequent polymerase chain reaction. Anal. Biochem. 282, 259–262.
[21] Huang, X. and Madan, A. (1999) CAP3: A DNA sequence assembly program. Genome Res. 9, 868–877.
[22] Gish, W. and States, D.J. (1993) Identification of protein coding regions by database similarity search. Nat. Genet. 3, 266–272.
[23] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
[24] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
[25] Leonhardt, N., Kwak, J.M., Robert, N., Waner, D., Leonhardt, G. and Schroeder, J.I. (2004) Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell 16, 596–615.
[26] Vrinten, P.L., Nakamura, T. and Kasha, K.J. (1999) Characterization of cDNAs expressed in the early stages of microspore embryogenesis in barley (*Hordeum vulgare*) L. Plant Mol. Biol. 41, 455–463.
[27] Kumlehn, J., Kirik, V., Czihal, A., Altschmied, L., Matzk, F., Lörz, H. and Bäumlein, H. (2001) Parthenogenetic egg cells of wheat: cellular and molecular studies. Sex. Plant Reprod. 14, 239–243.
[28] Gerke, V. and Moss, S.E. (2002) Annexins: from structure to function. Physiol. Rev. 82, 331–371.
[29] Clark, G.B., Dauwalder, M. and Roux, S.J. (1992) Purification and immunolocalization of an annexin-like protein in pea seedlings. Planta 187, 1–9.
[30] Clark, G.B., Dauwalder, M. and Roux, S.J. (1994) Immunolocalization of an annexin-like protein in corn. Adv. Space Res. 14, 341–346.
[31] Clark, G.B. and Roux, S.J. (1995) Annexins of plant cells. Plant Physiol. 109, 1133–1139.
[32] Carroll, A.D., Moyen, C., Van Kesteren, P., Tooke, F., Battey, N.H. and Brownlee, C. (1998) Ca^{2+}, annexins, and GTP modulate exocytosis from maize root cap protoplasts. Plant Cell 10, 1267–1276.
[33] Battey, N.H., James, N.C., Greenland, A.J. and Brownlee, C. (1999) Exocytosis and endocytosis. Plant Cell 11, 643–660.

[34] Proust, J., Houlne, G., Schantz, M.L., Shen, W.H. and Schantz, R. (1999) Regulation of biosynthesis and cellular localization of Sp32 annexins in tobacco BY2 cells. Plant Mol. Biol. 39, 361–372.
[35] Okamoto, T., Higuchi, K., Shinkawa, T., Isobe, T., Lorz, H., Koshiba, T. and Kranz, E. (2004) Identification of major proteins in maize egg cells. Plant Cell Physiol. 45, 1406–1412.
[36] Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M. and Laux, T. (2004) Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development 131, 657–668.
[37] Dong, J.Z. and Dunstan, D.I. (1999) Cloning and characterization of six embryogenesis-associated cDNAs from somatic embryos of *Picea glauca* and their comparative expression during zygotic embryogenesis. Plant Mol. Biol. 39, 859–864.
[38] Engel, M.L., Chaboud, A., Dumas, C. and McCormick, S. (2003) Sperm cells of *Zea mays* have a complex complement of mRNAs. Plant J. 34, 697–707.
[39] Engel, M.L., Holmes-Davis, R. and McCormick, S. (2005) Green sperm. Identification of male gamete promoters in Arabidopsis. Plant Physiol. 138, 2124–2133.